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STEM CELLS AND NANOTECHNOLOGY IN TISSUE ENGINEERING AND REGENERATIVE MEDICINE

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1.1 A BRIEF HISTORY OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Awareness of the natural regenerative capabilities of the human body dates back to ancient times. In Greek mythology, when Prometheus stole fire from the gods and gave it to the mortals, Zeus punished him by tying him to a rock and having an eagle peck away his liver, only to have it regrow and be eaten again the following day. Although the liver has significant natural regenerative capacity that seems to have been apparent for many ages, many other organs have a very limited ability to regrow after damage or removal. These limitations have spurred the development of regenerative approaches in the history of modern medicine, as clinicians and scientists continuously attempt to overcome the body's natural limitations.

The birth of whole-organ transplantation techniques has paved the way for modern developments in regenerative medicine and tissue engineering. Alexis Carrel, winner of the Nobel Prize in Physiology or Medicine in 1912 and the father of whole-organ transplant, was the first to develop a successful technique for end-to-end arteriovenous anastomosis in transplantation. In the 1930s, assisted by Charles

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Lindbergh, he developed a "perfusion pump" that allowed organs to be maintained outside the body during transplantation, a concept that has been more recently used in bioreactors for tissue engineering studies.[1,2]

The limitations of organ transplant because of immune reaction were recognized early. Gibson and Medawar found that application of a second skin allograft from the same donor resulted in faster rejection than the first, suggesting that the response may be immunologic.[3] Additional studies in dogs showed that allografts produced a mononuclear reaction to the transplanted organ.[4] Thus, to avoid the immune response, the first successful kidney transplant was performed in 1954 between identical twins.[5] It was not until the development of immunosuppressive drugs that transplantation from genetically different donors became feasible. Many advances have been made in the field of organ transplantation, including the development of immunomodulating therapies and significant reductions in the number of immunosuppressive drugs necessary after transplantation.[6,7] However, limitations because of organ or tissue availability and the continual need for chronic immunosuppression remain and have left physicians and scientists looking for a new approach that mitigates these issues. These efforts have resulted in the popularization of the fields of tissue engineering and regenerative medicine.

A succinct definition of regenerative medicine has been provided by Mason et al., stating that "regenerative medicine replaces or regenerates human cells, tissue or organs, to restore or establish normal function."[8] This broad definition can include the use of cell-based therapy, gene therapy, nonbiological devices, and tissue engineering strategies. Organ transplantation falls short in this definition because completely normal function is not possible given the need for continuous immune suppression.

Although the terms *tissue engineering* and *regenerative medicine* are sometimes used interchangeably, it is important to understand that tissue engineering falls under the umbrella of the regenerative medicine field but is not all encompassing. As defined by Langer and Vacanti, tissue engineering is "an interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function."[9] More specifically, tissue engineering uses a combination of cells, scaffolds, and bioactive factors in strategic combinations to direct the *in vitro* formation of new tissues or organs (Fig. 1.1). Regenerative medicine strategies, on the other hand, often rely on the body's natural processes to assist in the formation of new tissues after delivery of exogenous cells, scaffolds, or biomolecules.

As scientists have begun to unravel the complexity of biological processes on a cellular and molecular scale, the fields of tissue engineering and regenerative medicine have migrated toward applying this knowledge to control the interactions between cells and their environment, whether natural or assisted. Concomitantly, the applications of nanotechnology to biological processes have rapidly increased in recent years and hold the great promise for successful translation of bench research into the clinic.

In this introductory chapter, we will provide an overview of the central concepts in regenerative medicine and tissue engineering strategies. First, we will provide a

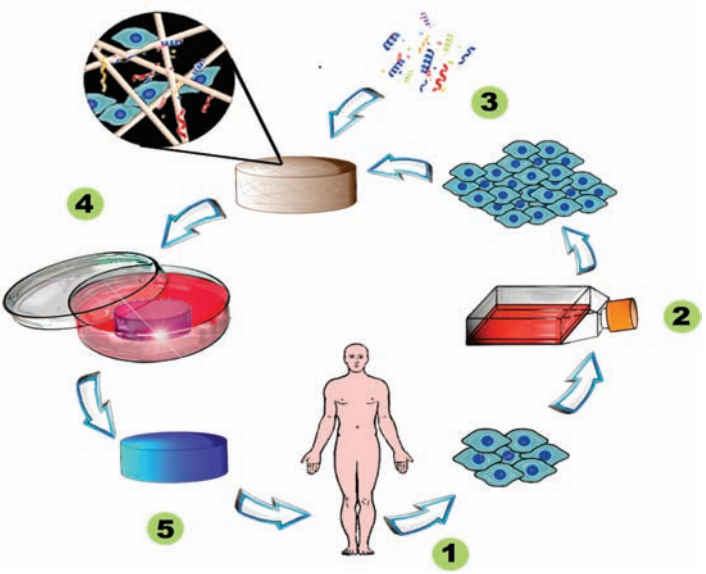

FIGURE 1.1 General schematic of tissue engineering strategy. (1) Cells are isolated from the patient and (2) expanded in 2D culture. (3) Expanded cells are then combined with various natural or engineered bioactive molecules (e.g., growth factors, nanoparticles, or DNA) into biocompatible scaffolds and (4) cultured *in vitro* under specific culture conditions to promote tissue formation. (5) Finally, functional tissue-engineered constructs are implanted into the donor to replace the damaged tissue.

brief overview of stem cells and their role in cell therapy strategies followed by a discussion of tissue engineering applications using stem cells in conjunction with biomaterials and bioactive factors. In particular, we will focus on the shift that has occurred in the field toward using nanoscale approaches that control cellular activities and tissue formation at the subcellular level.

1.2 INTRODUCTION TO STEM CELLS

To eliminate the need for immunosuppression, regenerative medicine and tissue engineering approaches have generally focused on using donor-derived autologous cells or cells that will not elicit an immune response. However, terminally differentiated cells are typically limited in their ability to proliferate, and it is therefore difficult to obtain sufficient cell number for regeneration of tissues. In addition, cells from tissues to be treated or replaced are likely to have undesirable defects that require structural repair. Because of this limited potential using differentiated cells, the use of stem or progenitor cells has become ubiquitous in the fields of tissue engineering and regenerative medicine. The characteristics that define stem cells—capacity for self-renewal, long-term proliferation, and the ability to differentiate into

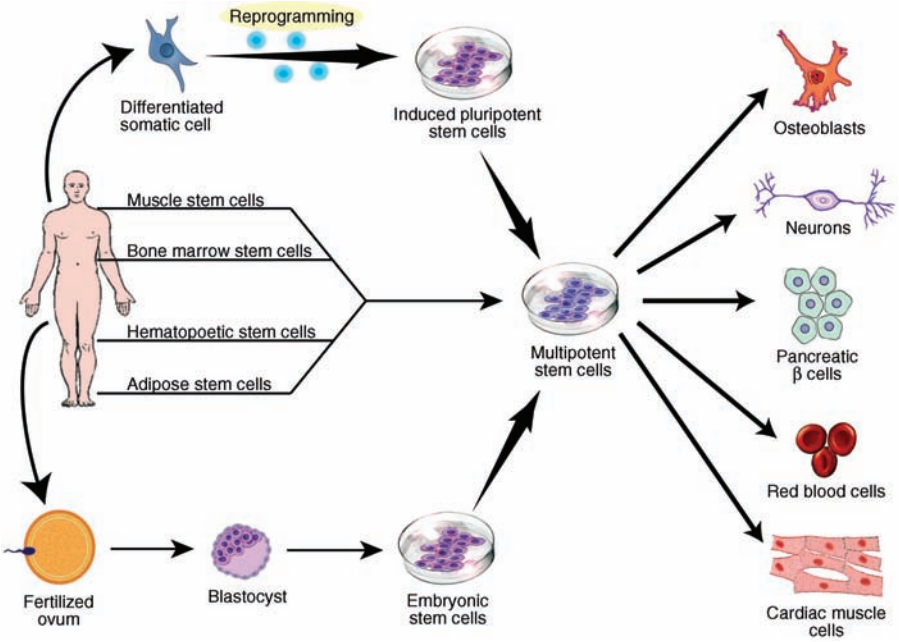

FIGURE 1.2 Isolation and differentiation of stem cells for tissue engineering and regenerative medicine. Multipotent stem cells can be obtained directly from various human tissues, including, but not limited to, bone marrow, blood, muscle, and adipose tissue. Pluripotent stem cells can be derived from the inner cell mass of the blastocyst (embryonic stem cells) or by reprogramming cells that were previously differentiated into a pluripotent state using specific factors (induced pluripotent stem cells). Multipotent stem cells can then be differentiated into many different cell types, only a few of which are shown here.

several different cell types—make them the optimal cell source for the development of new tissues and organs.

Stem cells are typically classified into two main groups: embryonic and adult. Figure 1.2 provides a summary of the origin and differentiation capacity of the different types of stem cells. Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocysts in the developing embryo before implantation. These cells are pluripotent, that is, they have the ability to differentiate into cells comprising all three germ layers and can be maintained in culture in an undifferentiated state for indefinite periods of time.[10] Although these characteristics make ESCs an attractive cell source for regeneration of tissues and organs, they have several limitations. First, ESCs present an ethical issue because embryos are destroyed in the process of obtaining the cells; second, because they are obtained from allogeneic sources, there is the possibility of an immune response, although research suggests that this response may be weaker than with traditional organ transplant;[11,12] third, because of their undifferentiated state, there is a possibility that ESCs can become tumorigenic and malignant.[13]

Adult stem cells, on the other hand, are isolated from postnatal tissues. They can also differentiate down multiple lineages but are more restricted in the types of cells that they can become and have more limited proliferation potential in comparison to ESCs. One advantage that adult stem cells have over ESCs is that they can often be obtained from the patients themselves, thereby eliminating the issue of immune rejection. However, if the tissue that contains the desired cells is diseased or has limited availability of stem cells (e.g., nervous tissue), it may not be possible to obtain autologous cells. Adult stem cells have been isolated from many different tissues, but one of the most widely used cell sources is bone marrow-derived mesenchymal stem cells (MSCs). These cells have been extensively characterized, and large numbers can be obtained with relative ease through a bone marrow biopsy. MSCs have the ability to differentiate into cartilage, bone, and fat, making them particularly useful for regeneration of musculoskeletal and connective tissues. In addition to their multipotent differentiation ability, MSCs have been shown to be hypoimmunogenic as well as immunosuppressive even after differentiation.[14,15]

Although adult stem cells are multipotent, unlike ESCs, they are somewhat lineage-restricted and typically only differentiate readily toward a few cell types and therefore are useful for a limited number of tissues. Recent advances, however, have led to the ability to reprogram somatic cells, including those that have undergone lineage specific differentiation, into an ESC-like state. Takahashi et al. showed that the retroviral-mediated expression of four nuclear transcription factors—Oct4, Sox2, Klf4, and c-Myc—resulted in cells with expression patterns and differentiation capacity cells similar to those of ESCs. These induced pluripotent stem cells (iPS cells) may allow scientists to overcome the ethical and immune rejection concerns of ESCs while retaining the increased proliferation and differentiation capacity that make the use of ESCs over typical adult stem cells desirable. Initial concerns that using viruses or vectors that integrate into the genome may lead to the development of tumors are being addressed by using transient or removable vectors or by directly delivering proteins.[16–21] Despite being isolated from the same individual, recent studies show that iPS cells may generate an immune response upon implantation,[22] suggesting that although these cells may have promise, a great deal more research must be done before using them in a clinical setting.

1.3 TISSUE ENGINEERING AND REGENERATIVE MEDICINE STRATEGIES

1.3.1 Cell Therapy

With the rising popularity of stem cell research, cell therapy has evolved as a potential treatment method for a variety of conditions. Cell therapy involves delivery of cells either into the bloodstream or directly into the tissue of interest. Although tissue engineering strategies combine cells with scaffold materials and bioactive factors before implantation, cell therapy relies critically on the interaction of the donor cells with host tissues to restore function.

The first widely used stem cell therapy in humans was bone marrow transplantation. In the late 1950s, Thomas et al. demonstrated that in two patients with leukemia, infusion of bone marrow from a healthy identical twin after total-body irradiation resulted in full reconstitution of the bone marrow and temporary remission.[23] Since then, bone marrow and hematopoietic stem cell transplant has become the standard treatment after myeloablation. In addition to repopulating the bone marrow after irradiation, allogeneic stem cell transplantation may further improve treatment outcomes through a graft-versus-tumor effect.[24,25]

As the use of stem cell transplants for reconstituting the immune system has increased, research has also expanded toward injection of other cell types into tissues, including solid organs. Clinical trials are currently underway using stem cells for regeneration of bone, cartilage, and cardiac tissue as well as for treating cancer, hematologic diseases, diabetes, and neurodegenerative disorders.[26] The first approved clinical trial using cells derived from ESCs for treatment of thoracic spinal cord injury was initiated in 2010, and additional phase I/II trials are ongoing for treatment of macular defects using retinal pigment epithelium derived from ESCs.[26]

The greatest limitations in stem cell therapy are the low survival rates of the injected cells and the inability to closely control the location of those cells that do survive. Studies examining the effects of the delivery of MSCs after myocardial infarction have shown that the majority of cells injected intravenously were eventually found in the lungs, spleen, and liver, with only a small percentage of the cells engrafted into the injured heart wall.[27–29] It is possible that some of these limitations could be overcome by injecting cells directly into the tissue; however, this increases the risk of further damage, and cell survival is still limited with this method.

Although cell therapies are currently limited by low percentages of cell engraftment, significant functional improvements are often still seen. Therefore, it has become widely accepted that it is not necessarily the differentiation and direct engraftment of the injected cells themselves that result in improved function but rather the autocrine and paracrine effects of the smaller number of cells that do survive and engraft. Therefore, although significant benefit may be derived through increasing survival and localization of stem cells after injection, understanding the mechanisms through which cells do provide benefit via trophic influences may eventually be of substantially greater value.

1.3.2 Tissue Engineering and Biomaterials

In tissue engineering, biomaterials serve as the scaffolding upon which cells build tissues. The definition of biomaterials has recently been revisited and is now interpreted as encompassing natural or synthetic materials that interact with biological systems.[30] The use of biomaterials in medicine has been around for centuries, with dental implants made from wood and contact lenses made from glass being some of the first common applications of biomaterials. Because early implants were designed to remain in place for long periods of time and little was known about the mechanisms behind the foreign body response, initial biocompatibility studies in the

1940s focused on determining which materials were the least chemically reactive. However, this changed with the development of applications in which it was desirable for the biomaterial to interact directly with the host tissue as well as degrade over time. Therefore, the definition of *biocompatibility* has become focused on materials having an "appropriate host response" rather than limiting the response.[31] Today, in addition to being biocompatible, biomaterials in tissue engineering applications have become increasingly sophisticated and are designed to meet several criteria. First, they should provide appropriate mechanical strength to ensure that the tissue can withstand the normal forces it experiences or perform its physical functions *in vivo*. Second, they must provide a compatible surface for cell attachment and appropriate topographic information. Third, they should ideally be designed to degrade over a length of time that is appropriate for the specific application, such that ultimately, the engineered tissue is able to approximate its native state.

Synthetic polymers have an advantage over natural polymers as biomaterials for tissue engineering because they may be produced using defined processes and have highly tunable mechanical and chemical properties to enhance biocompatibility. However, nature's biomaterial—the extracellular matrix (ECM)—already possesses the optimal properties to support cellular attachment and tissue growth, often in a tissue-specific manner. This has led tissue engineers to study in depth the structure and composition of the native ECM as well as investigate cell–material interactions with the goal of recreating this environment.

The native ECM is a complex and dynamic network of proteins that provides both structural and biochemical support to the cells it surrounds.[32] Rather than just serving as a passive scaffold, the ECM also provides important mechanical, topographic, and biochemical cues that can influence cell attachment, survival, shape, proliferation, migration, and differentiation.[33] The most abundant protein in the ECM is collagen, which makes up approximately 30% of the total protein in the human body. Mature collagen is a triple helix of three polypeptides that align and combine themselves to form collagen fibrils that are typically between 50 and 500 nm in diameter.[34,35] Other fibrous proteins such as fibronectin, laminin, and elastin are also present in significant quantities and influence the structural and mechanical properties of the tissue. In addition to the fibrous proteins, the ECM also contains glycoproteins as well as bound or entrapped growth factors that can significantly influence the properties of the tissue. Each component of the ECM influences cell behavior via specific interactions, often involving ligand-specific receptors on the cell membrane. Therefore, recapitulation of the structure of the native microenvironment using biomaterials with nanoscale features may provide the optimal biomimetic topographic structure for cells to form tissues with similar properties to the native tissue.

There are two levels of interactions that must be investigated to develop the optimal tissue engineered solution for clinical use: (1) the cell–material interactions *in vitro* after initial cell seeding and (2) the interactions of the tissue-engineered constructs with the host tissues after they are implanted. Although the ultimate goal of tissue engineering research is to develop a construct that can be implanted and function *in vivo*, it is imperative to first gain a thorough understanding of the cell–

scaffold interactions in well-defined *in vitro* environments. Tissue engineering research currently focuses on applying knowledge of the biological characteristics of native cellular and tissue microenvironment to the development of biomaterial-based constructs that mimic these behaviors when combined with cells.

1.3.3 Bioactive Factors in Tissue Engineering

In addition to the cell source and scaffolds, the use of bioactive factors is important for the optimization of tissue-engineered constructs. Although the ECM provides the structural component of the native tissue, it also contains soluble bioactive factors, such as growth factors and cytokines, whose signals direct aspects of cell behavior, including survival, proliferation, migration, and differentiation. *In vivo*, these biofactors are secreted by cells and exert their effects by binding to the receptors of target cells and stimulating signal transduction to alter gene expression. The effects of growth factors are dependent on the identity and state of the target cells as well as the structure and composition of the ECM. Different cell types can have different responses to the same growth factor, and one or more growth factors may induce the same downstream effects.

The behavior of growth factors is often modulated by the ECM, which can control the activity of growth factors in several ways. The effects of growth factors are dependent on the concentration of their active forms. Binding to components of the ECM, such as proteoglycans, can extend the stability of growth factors by protecting against proteolytic degradation and thus maintain effective concentrations. Alternatively, growth factors may be secreted in an inactive form and require cleavage or co-factor binding in the ECM to become activated.[36]

A large number of *in vitro* studies have successfully used growth factors to direct differentiation of stem cells down specific cell lineages. However, it may be difficult to effectively control differentiation *in vivo.* Specifically, intravenous administration of growth factors may be undesirable and largely ineffective since repeated infusions of high concentrations of growth factors would be required due to the short half-life of the factors, which may lead to negative systemic effects.[37] Therefore, the development of tissue engineering strategies that control the availability and limit the degradation of growth factors have gained increasing importance.

1.4 NANOTECHNOLOGY IN REGENERATIVE MEDICINE AND TISSUE ENGINEERING

1.4.1 Introduction to Nanotechnology

The human body is a complex, dynamic system with tissues and organs regulated at the subcellular level. Although there have been some successes in translating regenerative medicine and tissue engineering techniques to the clinic, they are few and are typically limited to a single or only a handful of patients. Progress in the field has been limited by the difficulty in recreating the complex interactions in

the native tissue environment that are needed to ensure functional tissue regeneration. As more is learned about the mechanisms that control tissue growth and formation, it has become clear that the answer most likely lies in controlling the cell behavior at the nanoscale. Although complete *in vitro* recreation of the *in vivo* environment remains a somewhat distant target, this may not be necessary for success. Instead, it is important that we aim to understand the interactions between cells and their native environment and apply this knowledge to the development of constructs that will jumpstart tissue formation down the correct path before allowing the most effective incubator—the human body—to take over and remodel the engineered cells or constructs into the optimal structure.

Because the individual chapters in this text will provide in-depth discussion of the different applications of nanotechnology in this field, we provide here a brief overview of past and current research that has used nanoscale approaches in tissue engineering and regenerative medicine.

1.4.2 Nano-Based Cell Tracking

Currently, the main application of nanotechnology in cell therapy is in cell tracking. Although histologic methods have traditionally been used to determine cell localization after implantation in animal models, these methods require harvesting, dissection and processing of the tissue, which is undesirable after implantation into humans. Recent developments in imaging technologies using nanoscale labeling methods that allow cells to be imaged *in vivo* and tracked in real time without compromising their function can improve understanding of cell fate after implantation and aid in future studies to improve cell engraftment and survival.

There are two main types of nano-based labeling techniques that have been investigated for noninvasive *in vivo* imaging: magnetic nanoparticles and fluorescent nanoparticles. Magnetic nanoparticles are commonly composed of superparamagnetic iron oxide (SPIO) particles 50–500 nm in diameter, which show enhanced contrast under magnetic resonance imaging (MRI).[38] SPIO particles have been successfully used for cell tracking in stem cell therapy studies in animals[39–41] and humans.[42,43] Although initially it was thought that SPIO particles did not affect cell behavior, recent studies suggest that exposure to magnetic fields after labeling may alter adipogenic and osteogenic differentiation capacity.[44] Further studies examining the effects on cells with SPIO particles under MRI are important before their clinical use.

Traditional fluorescent cell-labeling methods are limited by their lack of photostability, narrow excitation range, cell and tissue autofluorescence, and broad emission spectra.[45] Quantum dots, or Q-dots, are fluorescent nanoparticles with diameters that typically range from 2 to 5 nm and can be synthesized in any color.[46] Q-dots have greater photostability and a narrower emission profile than traditional fluorescent dyes, and they can be linked to specific proteins or DNA sequences to monitor specific cell behaviors.[47] Although *in vivo* studies using Q-dots are currently limited, early studies show that labeled cells can be tracked when injected into

mice.[48,49] However, there is some concern that they may alter cell function and differentiation, which may limit their use in a clinical setting.[46,50]

It is important to note that addition of genetic information to cells via viral particles, also known as gene therapy, is also a common method being tested in stem cell therapy to direct stem cell fate or to label cells for *in vivo* imaging. Viral vectors themselves may be considered a nanobiomaterial because they are typically engineered to deliver specific genes.[51] This important topic will not be explored in this chapter, which focuses instead on tissue engineering and regenerative medicine based on nonviral approaches.

1.4.3 2D Nanotopography

To examine the effects of topography on cells in a controlled environment, a large number of studies have fabricated substrates that contain microscale and nanoscale features on otherwise 2D surfaces. Results suggest that seeding cells onto substrates with micro- or nanoscale ridges, grooves, posts, or pits can affect cell attachment, shape, migration, proliferation, and differentiation.[52] Recently, mathematical algorithms have been used to generate a "chip" with thousands of random topographies and seeded with MSCs to examine the effects on proliferation and differentiation.[53] Application of these screens to determine the effects of varying topography may prove to be extremely valuable to understanding the optimal surfaces for controlling cell growth and differentiation.

In addition to physical topography, nanoscale manipulations of the biochemical structures can also strongly influence cell behavior. When biomaterials are either used in *in vitro* culture or implanted *in vivo*, the material surface becomes rapidly coated or opsonized with proteins before cell attachment. In both the native environment as well as on scaffolds, cells interact with ECM proteins via heterodimeric transmembrane receptors known as integrins. When integrins come into contact with specific peptide sequences on ECM molecules, they cluster and recruit other proteins to form the focal adhesion complex and signal to downstream effectors, which then influence cellular behavior.[54–56]

One of the most common integrin binding sequences is the arginine-glycine-aspartic acid (RGD) sequence. By modifying a substrate with the RGD peptide, cell adhesion, proliferation, and migration can be altered.[57] Because obtaining substantial quantities of purified proteins is difficult and may cause adverse host immune responses, the use of short synthetic peptides that are not recognized by the host's immune system but still contain functional domains to modulate cell adhesion to scaffolds is an attractive method for tissue engineering. However, since the concentration and distribution of cell adhesion peptides are both likely to significantly affect cell behavior, it is important to optimize these parameters for specific applications. For instance, it has been shown that clustering of RGD molecules into groups of nine improved cell motility compared with groups of five or individual molecules, independent of overall RGD concentration.[58]

Although the RGD molecule is a common receptor for many different integrins, integrin specificity for full-length proteins is determined by the configuration of

multiple regions within the protein. This is important because each integrin exerts specific intracellular downstream effects that differentially influence cell behaviors. For example, it has been shown that varying surface chemistry can influence whether cell attachment is mediated through binding to integrin $\alpha_5\beta_1$ or integrin $\alpha_v\beta_3$.[59,60] One study showed that when RGD alone was presented to cells, or with its synergy site PHSRN, adhesion was mediated by $\alpha_V\beta_3$. However, when a longer purified recombinant region of the fibronectin protein was used, osteoblast adhesion occurred through integrin $\alpha_5\beta_1$, suggesting that the tertiary structure of the protein may be important to consider when designing scaffolds for tissue engineering that specifically control cell behavior.[61]

Studies using surfaces modified to have nanoscale features or express specific cell-adhesion peptides provide a highly organized way of examining cell behavior with topographic and biochemical cues and give important insights into the interactions of cells with textured surfaces. However, these substrates are limited in their complexity compared to the native tissue environment. The textured surfaces only provide cues to the portion of the cell that is in contact with the surface and essentially limit cell motility to two dimensions. Although this is relevant for epithelium and vasculature endothelium, for most other tissues, it does not closely mimic the native environment. Additionally, the time course of these studies is typically short, making it impossible to examine key activities in tissue formation, such as ECM deposition and modification. Therefore, tissue engineering studies aimed at building a functional tissue *in vitro* typically focus on developing and using porous scaffolds where cells are surrounded on all sides by the scaffold material, similar to the native ECM, and the constructs are maintained in culture for several weeks to allow time for protein deposition and remodeling.

1.4.4 3D Nanoscaffolds

Extensive studies have shown that there are clear differences in cell behavior and tissue formation on flat surfaces compared with 3D ECM scaffolds and that these differences occur at the protein and subprotein level.[62–65] Furthermore, these differences are present even when the 2D scaffold has the same chemical and molecular composition.[63] These findings, in combination with the evidence that nanoscale topography affects cell behavior, have pushed scientists to use tissue-engineering approaches that mimic the 3D submicron structure of the native ECM. The currently available techniques for scaffold fabrication do not approach the complexity of the native ECM structure, nor can the environment be as closely controlled as with the 2D nanotopography described earlier. However, this may not be necessary because scaffolds in tissue engineering studies are designed to act primarily as a temporary structure to support cells until they are able to synthesize their own functional ECM. Therefore, the goal in successful application of 3D tissue engineered scaffolds is to identify and enhance key components that will provide the appropriate signals to cells to generate a functional tissue that can be translated into clinical use.

Nanobiomaterial constructs can be made using a wide variety of materials and methods to create scaffolds with many different structural and chemical properties that can be tailored to the specific application. As described earlier, the native ECM is composed predominantly of nanofibrous proteins. Therefore, efforts to recreate an environment similar to the original tissue have heavily focused on the development of fibrous scaffolds. A brief overview of the most common methods of synthesizing nanofibrous scaffolds is provided next. Other chapters in this volume present more in depth descriptions of these techniques and their applications in tissue engineering.

1.4.4.1 Phase Separation Thermally induced phase separation (TIPS) is a technique that is particularly useful for generating scaffolds with a specific pore size. In this method, the temperature of the polymer solution is adjusted to a point at which a "polymer-rich" and a "polymer-poor" phase is generated. The solvent is removed, and the polymer-rich phase solidifies, forming a porous solid structure, which is then freeze dried. Nanofibrous scaffolds with varying fiber diameters and pore sizes can be generated by adjusting the polymer concentration, the type of solvent, and the phase separation temperature.[66] Specifically, fibers ranging from 50 to 500 nm in diameter—similar to the size of native collagen—can be produced. Additionally, highly controlled interconnected macroporosity can be introduced by pouring the polymer solution over a negative wax mold, which is removed after solidification of the polymer.[67]

Nanofibrous scaffolds generated with TIPS have been shown to display increased attachment of seeded osteoblasts compared to solid wall scaffolds with similar macroporous architecture.[67] Additionally, mouse ESCs seeded in TIPS nanofibrous scaffolds and cultured under osteogenic conditions expressed higher levels of osteogenic genes and showed immunohistochemically detectable greater immunofluorescence than cells cultured on flat surfaces.[68]

TIPS is a simple and reproducible method for scaffold synthesis that does not require any specialized equipment. Additionally, it can be used to form complex 3D shapes that can be designed for an individual patient (Fig. 1.3).[67] However, it also has some important limitations. First, the technique can only be used with a limited number of polymers, and second, it would be difficult to scale up to a commercial level.[69]

1.4.4.2 Self-Assembly Another method of generating nanofibrous scaffolds that mimic the structure of the ECM is through self-assembly of peptide amphiphiles (PAs). This is a bottom-up approach that mimics natural processes such as nucleic acid and protein synthesis. PAs are short peptide structures that spontaneously aggregate into cylindrical micelles approximately 5–8 nm in diameter and 1 μm in length. This process occurs through noncovalent bonds under specifically tailored conditions. The peptides are typically composed of a hydrophobic alkyl chain tail, which forms the inside core of the fiber, and a hydrophilic head composed of epitopes, such as RGD, typically found in the native ECM that face outward, and interact with cells or other components of the ECM (Fig. 1.4). Other structural domains positioned between the tail and head regions of the PA function to stabilize

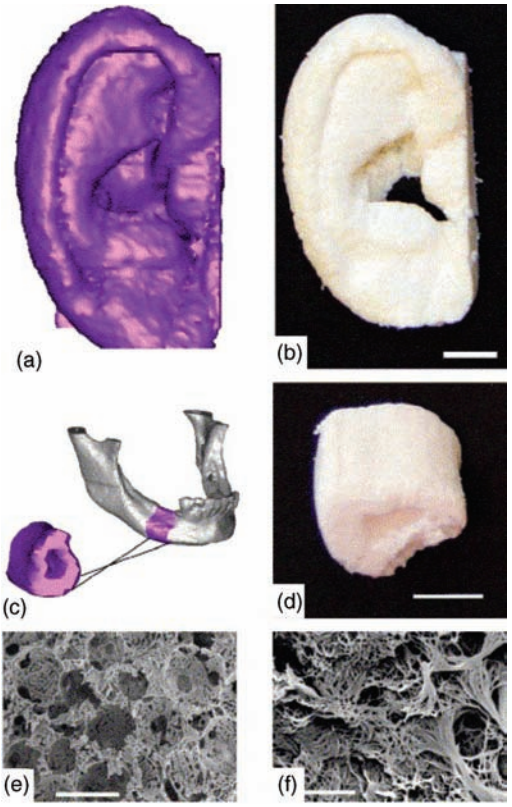

FIGURE 1.3 Scaffolds created using thermally induced phase separation derived from three-dimensional reconstructions of computed tomography (CT) scans. (a) Human ear reconstructed from histological sections and (b) the resulting nanofiber scaffold (scale bar = 10 mm). (c) Human mandible reconstruction from CT scans and (d) resulting nanofiber scaffold (scale bar = 10 mm). (e) Scanning electron micrographs showing interconnected spherical pores within mandible segment (scale bar = 500 μm) and (f) nanofiber pore morphology within a single pore (scale bar = 5 μm). Reprinted with permission from Ref. [67].

the network through hydrogen bonds and include regions that contain charged amino acids that control the solubility of the PAs under different pH conditions.[70] These structural features are particularly useful for generating injectables that are prepared under conditions that prevent self-assembly but are induced to undergo rapid self-assembly into a nanofiber network upon exposure to physiological pH. Bioactive factors such as DNA, drugs, or other proteins may be mixed into the unassembled solution for encapsulation into the fibers upon assembly and then released into the surrounding environment upon degradation.

Self-assembly of PAs can be used to generate a large variety of nanostructures with specifically tuned biochemical and degradation properties. For example, to promote mineral deposition for bone formation, phosphoserine residues have been

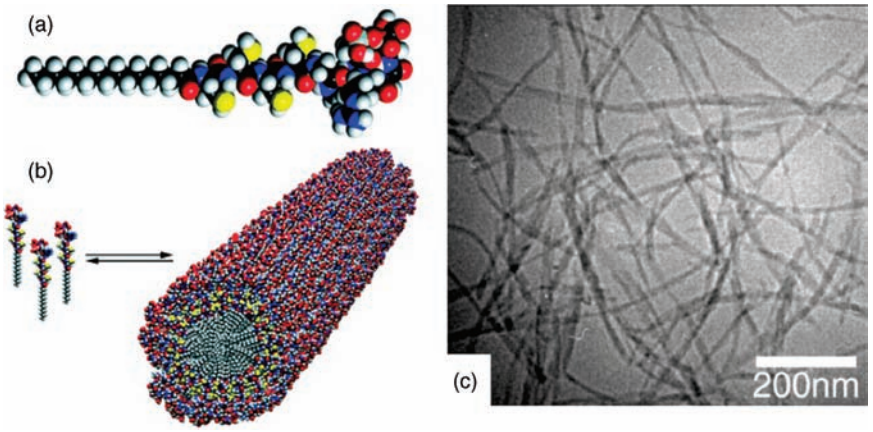

FIGURE 1.4 General structure of self-assembled peptide amphiphile (PA). (a) Molecular model of the PA showing the overall shape of the molecule. The *narrow gray area* represents the hydrophobic alkyl tail and the thicker head region is composed of hydrophilic amino acids containing functional groups that can provide signals to the cells to influence their behavior. (b) The PAs self-assemble into nanofibrous structures upon exposure to physiological conditions with the hydrophobic tail in the core and the head region facing the outside to interact with cells. (c) Vitreous ice cryotransmission electron microscopy image of hydrated PA fibers (scale bar = 200 nm). Modified with permission from Ref. [70].

incorporated into PAs with adhesion-promoting RGD head sequences. These phosphoserine residues serve as a template for nucleation of hydroxyapatite crystals that align along the long axis of the nanofibers, similar to the native bone structure.[71] The RGD peptides on the outer region of the PA promote cell adhesion. Other studies have shown that the use of self-assembled PAs can promote neural regeneration[72] as well as angiogenesis.[73] This technique not only provides biological cues to induce tissue formation but also mimics the basic steps of ECM biosynthesis. However, this method of nanofiber synthesis is quite complex and of relatively low yield and may not be suitable for large-scale tissue engineering applications.[74]

1.4.4.3 Electrospinning Electrospinning has recently become the most commonly used method for the fabrication of nanofibrous biomaterials. This method involves the application of a high electric field to a polymer solution delivered at a constant rate through a needle. At a high enough voltage, the charge on the polymer overcomes the surface tension of the solution and causes emission of a fine polymer jet. This jet undergoes a whipping process, and the fibers are further elongated as the solvent evaporates and fibers are deposited on a grounded collector (Fig. 1.5). Both natural and synthetic polymer scaffolds have been successfully created using the electrospinning method. The ability to generate three-dimensional scaffolds with tailored architecture, mechanical properties, and degradation characteristics has made electrospinning a popular method in tissue engineering applications. Altering parameters during the electrospinning process, such as polymer concentration, flow

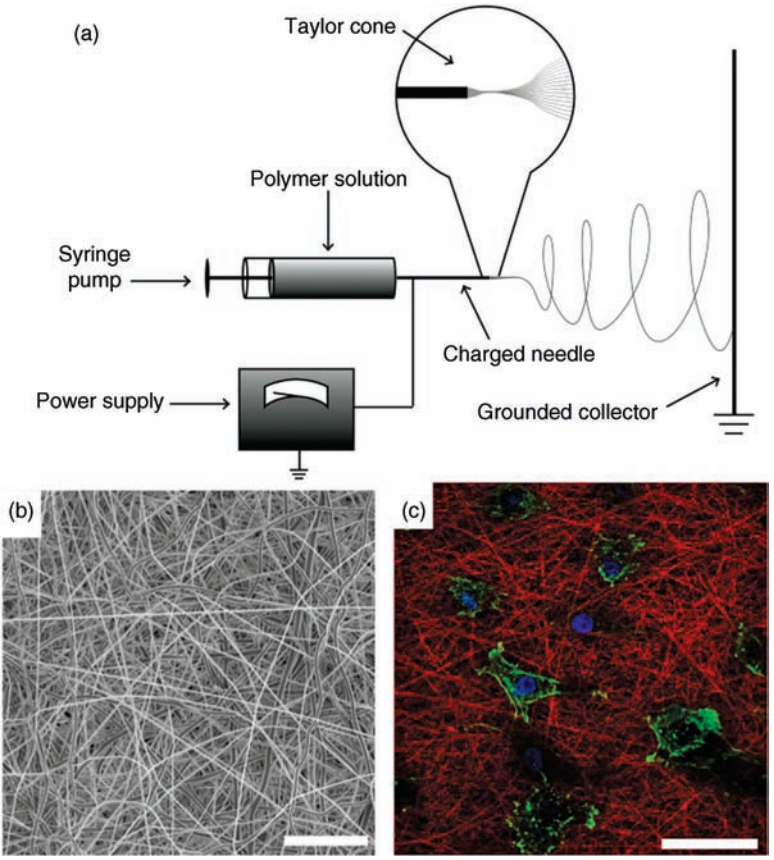

FIGURE 1.5 Fabrication of electrospun nanofibers. (a) General electrospinning setup consisting of syringe filled with polymer solution that is pumped through a needle charged with a high-voltage power supply. When the electrostatic forces between the collector and the solution overcome the surface tension of the solution, the solution is pulled out of the Taylor cone into fine fibers that are deposited on the grounded collector. (b) Scanning electron microscopic image of randomly arranged poly(ε-caprolactone) (PCL) nanofibers formed by electrospinning (scale bar = 20 μm). (c) Fluorescent image of mesenchymal stem cells seeded on nanofibers. Green, cells (membrane label); red, nanofibers; blue, cell nuclei (DAPI stain); scale bar = 20 μm.

rate of the solution, and voltage applied, can generate fibers ranging from approximately 100 nm to several micrometers in diameter. Whereas scaffolds with aligned fibers can be created by collecting fibers on a drum or mandrel rotating at high speeds, randomly oriented fibers are generated on slowly rotating or stationary collectors.

Various polymers have been used in electrospinning of nanofiber scaffolds. These include synthetic polymers such as poly(L-lactic acid) (PLLA),[75–78] poly (ε-caprolactone) (PCL),[79–85] and polyurethane,[86] as well as natural polymers such as

collagen,[87–90] elastin,[87,91] silk fibroin,[92–94] dextran,[95] and chitosan.[96,97] Because the synthetic polymers typically used in electrospinning are hydrophobic and lack biologically active functional groups, they are often modified either physically or chemically after the electrospinning process to increase their hydrophilicity and ability to interact with cells and biomolecules.

Plasma treatment, similar to that performed on tissue-culture polystyrene, can generate functional carboxyl or amine groups on the surface of the fibers. This has been shown to enhance cell attachment and proliferation either alone[98,99] or via the coating of the functionalized fiber with a natural ECM protein such as collagen[100,101] or gelatin.[102] Wet chemical etching methods may provide more homogeneous functionalization in thicker scaffolds because plasma etching can only penetrate the outer surface of a thicker scaffold. This method typically involves NaOH hydrolysis or aminolysis of the polymer, breaking the ester bond at random points and creating a hydroxyl or amino group, respectively.[103] One study demonstrated that esophageal epithelial cells seeded on aminolyzed poly(L-lactide-*co*-caprolactone)(PLCL) coated with fibronectin exhibited higher collagen type IV synthesis than those seeded on the unmodified polymer, suggesting that this method may be useful in tissue engineering studies.[104]

Composite scaffolds formed from co-electrospinning of different polymers have been used to control the mechanical as well as structural properties of the scaffold. Perhaps the biggest challenge using the electrospinning method is that the pore size is typically much smaller than the diameter of a typical cell, a property that makes cell and nutrient infiltration into the middle of the scaffold difficult. Several methods have been used to overcome this problem, including spinning of mixed microfiber and nanofibers scaffolds,[105] as well as using water-soluble polymers (i.e., polyethylene oxide [PEO]) in combination with slower-degrading materials (i.e., PCL), that can be quickly dissolved after spinning, leaving the nonsoluble, slower-degrading polymer behind with larger pore sizes.[106–108]

To more closely tailor the properties of a scaffold—including biologic, mechanical, and degradation characteristics—researchers have begun to combine two or more different components within a single scaffold. This can be done before electrospinning by mixing several polymers within a single solution, which results in a single fiber containing each component or by electrospinning multiple solutions of polymers onto the same collector, thereby creating a scaffold with multiple fiber types.

Although natural polymer scaffolds composed of ECM proteins such as collagen and elastin show increased cellular response, when used alone, they lack sufficient mechanical properties to function in the *in vivo* setting. Combining ECM derived from urinary bladder matrix with poly(ester-urethane)urea, Stankus et al. were able to develop electrospun scaffolds with improved mechanical and biological properties than possible using the individual polymers alone.[109] Similarly, Lee et al. mixed collagen and elastin with several biodegradable synthetic polymers to develop scaffolds to use as vascular grafts.[110]

Some polymers cannot be dissolved in the same solvent, therefore limiting the options for combining several different polymers within the same solution.

Additionally, it may be desirable to have fibers of different dimensions or mechanical properties within the same scaffold. This has led to the development of multi-jet electrospinning in which different polymer solutions can either be electrospun at the same time to generate a homogeneous mixed scaffold or sequentially to generate a layered scaffold.[111] Baker et al. co-electrospun three different solutions containing polymers with varying degradation rates and mechanical properties to develop a scaffold that allowed for both improved cellular infiltration by increasing pore size as well as more closely mimicked the properties of the native tissue.[112]

Electrospinning is a relatively simple, cost-effective technique that has shown significant potential in studies aimed at repair of many different types of tissues. When seeded with stem cells, nanofiber scaffolds have been shown to enhance differentiation toward many different cell types, including bone, cartilage, cardiac and skeletal muscle, blood vessels, and nerve.[113,114]

1.4.5 Growth Factor Delivery

Although the scaffold structure plays an essential role in controlling cell behavior, chemical or biological modulators of cell activity and phenotype heavily influence tissue formation both *in vitro* and *in vivo*. In native tissues, growth factors provide specific signals to cells that direct cell activities, including cell migration, proliferation, and differentiation. The effects of growth factors are quite complex and are dependent on the concentration of the growth factor, phenotype of the cells acted on by the growth factor, and functional characteristics of the specific cell receptor interacting with the growth factor.

In vitro tissue engineering studies often supply relevant growth factors in the culture medium to induce cellular differentiation. However, because most growth factors have very short half-lives, in order to maintain long-term signaling when tissue engineered constructs are implanted *in vivo*, it is important to develop a delivery system that can provide sufficient concentrations of specific factors over the desired period of time, preferably at specified rates. Nanoscale techniques for growth factor delivery have typically focused on two basic methods: (1) immobilization of the growth factor on the surface of a substrate or (2) encapsulation of the growth factor within a degradable delivery system.

Growth factors can be immobilized onto a material surface through either physical adsorption or through covalent linkage. Although simple physical adsorption is limited in its effectiveness because of competition by other proteins with higher affinity for the polymer,[115] successful noncovalent adsorption onto a nanomaterial has been accomplished by mixing heparin into a synthetic polymer solution that is then electrospun into nanofibers. Heparin is a sulfated glycosaminoglycan that has a strong affinity for a number of growth factors, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β). In one study, low-molecular-weight heparin was conjugated to a poly(ethylene glycol) (PEG) carrier and electrospun with either PEO or poly(lactide-*co*-glycolide) followed by successful

adsorption of bFGF.[116] Another study demonstrated that bFGF and EGF adsorbed onto PLLA nanofibers coated with covalently linked heparin maintained their activity and induced neural differentiation and axon growth in human ESCs, but simple adsorption of the growth factors did not have an effect on the cells.[117]

Although noncovalent adsorption is useful if presence of the growth factor is only needed initially, covalent linkage of growth factors to nanofibers is typically preferred for tissue engineering applications because of the slower release profile, which is dependent on the rate of degradation of the polymer to which it is attached. In one study, amine-terminated PCL–PEG block copolymers were electrospun and EGF was covalently immobilized on the PEG-linked amine. In a mouse diabetic wound model, EGF conjugated nanofibers showed enhanced healing compared with unconjugated EGF and nanofibers alone, as well as upregulated EGF receptor expression on the cells in the wound area.[118]

To regulate the release of growth factors from a surface, some investigators have examined the effects of encapsulating the growth factors in nanoparticles, which are then adsorbed onto a nanofiber surface. In one study, PLGA nanoparticles containing platelet-derived growth factor (PDGF) were immobilized onto nanofibrous PLLA scaffolds.[119] Growth factor release from nanoparticles was prolonged when they were immobilized to scaffolds compared with free nanoparticles, and bioactivity was retained over a 14-day period, suggesting that this method may be useful for delivery of growth factors to influence cellular activity in a tissue engineered construct. Additionally, the amount and rate of release of growth factors can be controlled by altering one or more parameters, including the biodegradability of the nanoparticles, the molecular weight of the polymers used, the ratio of lactic to glycolic acid, or the amount of growth factor encapsulated within the nanoparticle.

Incorporation of growth factors directly into the fibers of scaffolds has also shown promise as a method for sustained delivery, although this approach can alter the degradation and mechanical properties of the scaffold and must be considered during synthesis.[120] Another challenge in this strategy is that exposing growth factors to the organic solvents used to generate polymer solutions can denature the growth factors.[121] This is typically solved by incorporation of a hydrophilic additive, such as PEO or bovine serum albumin (BSA), which minimizes the contact between the protein and the organic solvent. Studies have demonstrated that electrospinning solutions containing nerve growth factor (NGF) with BSA and either PCL or PLCL can produce nanofibers that release active NGF over several weeks and promote neurite outgrowth when seeded with PC-12 cells.[122,123] Bone morphogenetic protein-2 (BMP2) was incorporated into silk scaffolds with PEO and demonstrated increased osteogenic differentiation of MSCs and calcium deposition compared with scaffolds without BMP2.[124] A novel method of nanoparticle synthesis using sugar molecules to protect proteins from degradation under harsh environments as well as allow for sustained release of the protein of interest has recently been developed. These nanoparticles have excellent storage stability and can be used with almost any protein or polymer of interest, making them particularly attractive as a method for delivering bioactive factors within a scaffold.[125]

Coaxial electrospinning has also been investigated as a method of growth factor delivery. In this method, two solutions are pumped through concentric needles to form fibers containing an outer shell and inner core of different components. Placing the solution containing the growth factor on the inside of the fiber reduces the potential for denaturation by the organic solvent used to dissolve the outer polymer. One study compared the release of bFGF from electrospun fibers that were prepared by direct blending the bFGF into the polymer solution and by coaxial electrospinning with bFGF in the core of the fiber.[126] Both methods resulted in increased attachment, proliferation, and differentiation of seeded bone marrow stem cells compared with cells on fibers without bFGF. However, coaxial electrospinning resulted in a slower release profile of bFGF compared with the blended method. Another study showed that the protein release rate from a coaxial electrospun fiber could be increased by increasing the feed rate of the core solution or by adding a polymer with a faster rate of degradation (i.e., PEG) to the outer shell solution.[127] The ability to tailor both the mechanical and degradation properties of the scaffold as well as control the release rate of growth factors from the scaffold makes this an attractive method for use in future tissue engineering studies.

1.5 CONCLUSIONS

The ultimate goal in regenerative medicine and tissue engineering is to develop technologies to repair or replace tissues without the complication of chronic immunosuppression and dependence on organ donors. The key to success is understanding of how native tissues function and applying this information to establish the proper combination of cellular, structural, and chemical components that will allow for functional tissue development. Although perfect mimicry of the complex tissue structure found in nature is unlikely to be reached soon, it is critically important to gain a fuller understanding of how cells receive the signals needed to achieve the appropriate phenotype and to form functional tissues once implanted *in vivo*. It is increasingly apparent that such investigations will need to transcend the tissue, or even the cellular level, and take into consideration nanoscale phenomena that control interactions between cells, scaffolds and bioactive substances. Significant advances have been made both in deciphering the biology behind cell–matrix interactions as well as generating artificial ECM and controlling stem cell fate in the laboratory; however, significant improvements remain necessary to make regeneration of tissues a widespread clinical option. This chapter provides an abbreviated overview of the exciting developments and conceptual and practical challenges in cell-nanomaterial biology and engineering that is explored in depth in the chapters of this book.

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